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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2008, p. 3779–3782 Vol. 52, No. 10
0066-4804/08/$08.00+0 doi:10.1128/AAC.01665-07
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Edelfosine Induces an Apoptotic Process in *Leishmania infantum* That Is Regulated by the Ectopic Expression of Bcl-X_L and Hrk[▽]

Juan Fernando Alzate,[1] Andrés Arias,[2] Faustino Mollinedo,[3] Eva Rico,[2] Janis de la Iglesia-Vicente,[3] and Antonio Jiménez-Ruiz[2]*

Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia[1]; Departamento de Bioquímica y Biología Molecular, Campus Universitario, Universidad de Alcalá, Alcalá de Henares, Madrid 28871, Spain[2]; and Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, Consejo Superior de Investigaciones Científicas-Universidad de Salamanca, Campus Miguel de Unamuno, Salamanca E-37007, Spain[3]

Received 25 December 2007/Returned for modification 25 March 2008/Accepted 7 July 2008

The alkyl-lysophospholipids edelfosine and miltefosine induce apoptosis in *Leishmania infantum* promastigotes. The finding that edelfosine-induced cell death can be regulated by the ectopic expression of the antiapoptotic and proapoptotic members of the Bcl-2 family of proteins Bcl-X_L and Hrk suggests that this process is similar to apoptosis in eukaryotic cells.

Miltefosine, edelfosine, and several other alkyl-lysophospholipids originally developed as anticancer agents have proved to be effective antileishmanial drugs (4, 7). An increasing number of reports have shown that culture saturation (3), heat shock treatment (1, 5), and exposure to chemotherapeutic agents such as glucantime (8) and miltefosine (6, 9) induce features of programmed cell death (PCD) on *Leishmania* parasites. To gain further insight into this process, we have investigated whether proteins that regulate apoptosis in higher eukaryotes have any effect on edelfosine-induced PCD in *Leishmania infantum*.

Exponentially growing (2×10^6 parasites/ml) *L. infantum* (M/CAN/ES/96/BCN150 MON-1) promastigotes were subjected to incubation with increasing concentrations of either edelfosine or miltefosine for 24 h, and the percentage of dead parasites was evaluated by flow cytometry after the parasites were stained with 5 μM propidium iodide (PI) (1). The increase in drug concentrations correlated with the percentages of PI staining-positive parasites, which was an indication of the cytotoxic effects of the drugs (Fig. 1A and B). The estimated 50% lethal doses were 27 μM ($R^2 > 0.99$) for edelfosine and 47 μM ($R^2 > 0.99$) for miltefosine. The DNA content in the drug-treated parasites was analyzed by flow cytometry (1). Both drugs induced a concentration-dependent process of DNA degradation, as shown by the progressive increase in the percentages of hypodiploid cells (Fig. 1C and D). The results also reveal that edelfosine has greater potency than miltefosine against *L. infantum* promastigotes.

Changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$) were analyzed by flow cytometry after staining of the parasites with tetramethylrhodamine methyl ester (TMRM) (2). As already shown for heat-induced cell death (1), edelfosine or miltefosine treatment causes a nonhomogeneous effect on the $\Delta\Psi_m$ of the parasites (Fig. 1E and F). After 24 h, the parasites can be divided into two populations according to their mitochondrial status: the first one is composed of parasites with a reduced $\Delta\Psi_m$, and the second one is composed of a population in which the parasites show a clear increase in $\Delta\Psi_m$ compared to that for the untreated controls. This increase in $\Delta\Psi_m$ can be observed as soon as 30 min after drug treatment (data not shown) and may be interpreted as a strategy that the cell uses to obtain enough energy to develop the apoptotic process. The observed decrease in $\Delta\Psi_m$, together with the presence of a sub-G_1 peak in the cell cycle analysis, is suggestive of a death process similar to that of apoptosis in response to edelfosine or miltefosine. The induction of apoptosis following miltefosine treatment has already been reported in *Leishmania donovani* (6, 9, 10).

Apoptosis in higher eukaryotes is regulated by members of the Bcl-2 family of proteins (11). When parasites transfected with a pX63-Neo vector containing the *bcl-X_L*-coding sequence were treated with edelfosine, significant decreases in the number of hypodiploid cells (Fig. 2A) and the number of cells present in the population with a low $\Delta\Psi_m$ (Fig. 2B) were

TABLE 1. Measurement of apoptosis by TUNEL technique[a]

Cells	Treatment	% TUNEL assay-positive cells
L. infantum wild type	Control	0
L. infantum wild type	40 μM edelfosine, 24 h	42.5 ± 4.7
L. infantum pX63	Control	0
L. infantum pX63	40 μM edelfosine, 24 h	47.4 ± 5.2
L. infantum Bcl-X_L	Control	0
L. infantum Bcl-X_L	40 μM edelfosine, 24 h	26.7 ± 3.1
L. infantum Hrk	Control	0
L. infantum Hrk	40 μM edelfosine, 24 h	92.5 ± 8.3

[a] Apoptosis was measured by the TUNEL technique with untreated control cells and cells treated with 40 μM edelfosine for 24 h. Wild-type parasites and parasites transfected with the empty expression vector pX63 (*L. infantum* pX63), pX63–*bcl-X_L* (*L. infantum* Bcl-X_L), or pX63-*hrk* (*L. infantum* Hrk) were used. The data are shown as the means ± standard deviations (n = 3).

* Corresponding author. Mailing address: Departamento de Bioquímica y Biología Molecular, Campus Universitario, Universidad de Alcalá, Alcalá de Henares 28871, Madrid, Spain. Phone: (34)918855109. Fax: (34)918854585. E-mail: antonio.jimenez@uah.es.
▽ Published ahead of print on 21 July 2008.

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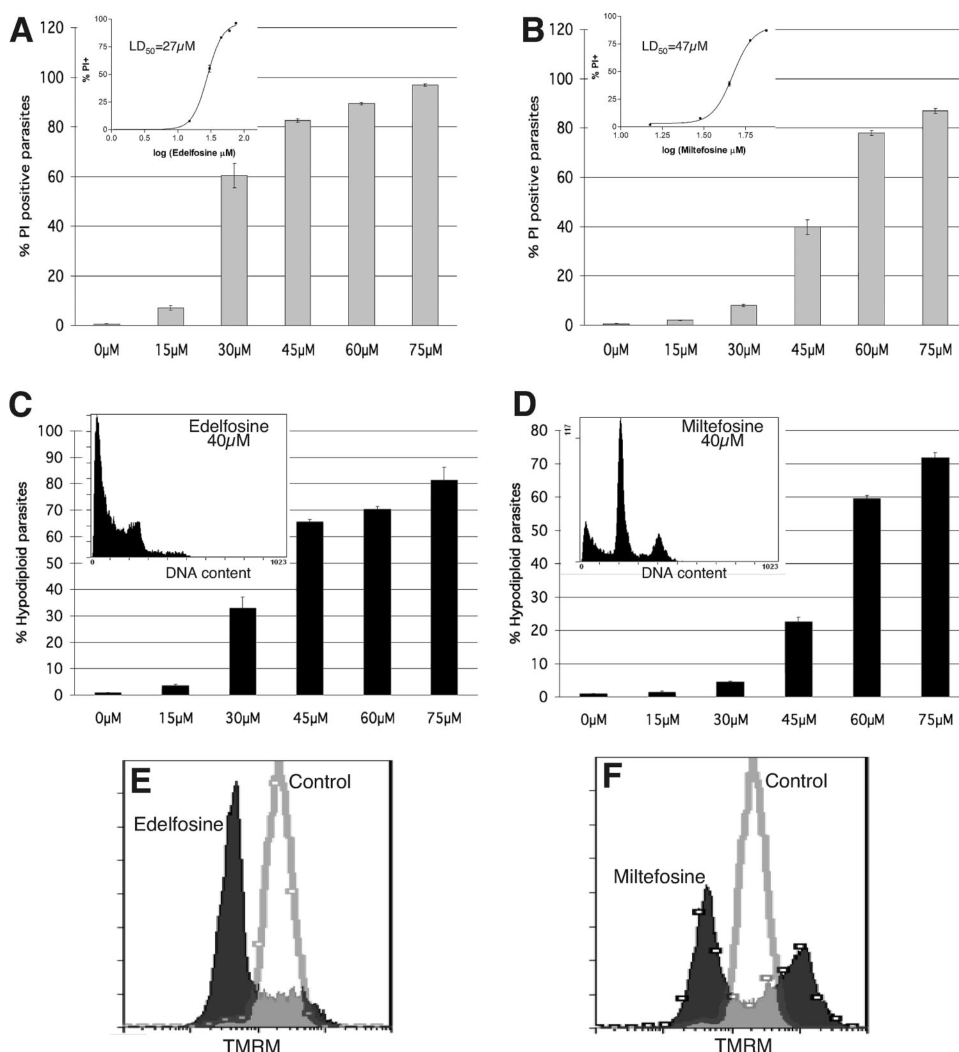

FIG. 1. Leishmanicidal effects of edelfosine and miltefosine. (A and B) Percentages of PI-positive *L. infantum* promastigotes after 24 h of treatment with increasing concentrations of edelfosine (A) or miltefosine (B); (C and D) percentages of hypodiploid promastigotes after 24 h of treatment with increasing concentrations of edelfosine (C) or miltefosine (D); (E and F) monoparametric histograms comparing the relative TMRM-derived fluorescence of control and 45 μM edelfosine-treated (E) or 45 μM miltefosine-treated (F) promastigotes. LD_{50}, 50% lethal dose.

observed. Our results clearly indicate that both apoptotic processes are partially reverted by Bcl-X_L expression.

To check whether a proapoptotic member of the Bcl-2 family could also modify the response of the cell population to edelfosine, *L. infantum* promastigotes were transfected with a pX63-Neo vector containing the *hrk*-coding sequence. Significant increases in the percentage of hypodiploid parasites (Fig. 2A) and the number of promastigotes with low $\Delta\Psi_m$ values (Fig. 2B) were observed when these transfected promastigotes were treated with 40 μM edelfosine for 24 h.

The effect of Bcl-X_L or Hrk expression was further confirmed by the terminal deoxyribonucleotidyltransferase-mediated dUTP-biotin nick-end-labeling (TUNEL) method (Fig. 2C). Statistical analysis of the images (Table 1) revealed that the percentages of fluorescent cells in nontransfected parasites and in parasites transfected with an empty vector (pX63) were similar (42.5% and 47.4%, respectively), whereas only 26.7% of the parasites expressing the antiapoptotic Bcl-X_L was TUNEL assay positive. On the other hand, 92.5% of the parasites expressing the proapoptotic Hrk were positive for staining by the TUNEL assay, which confirms the proapoptotic effect of the expression of this protein in *Leishmania* parasites.

We have previously shown that Bcl-X_L is able to partially revert heat shock-induced cell death in *L. infantum* promastigotes (1). The prosurvival activity of this protein was also confirmed in the present work. On the other hand, the expression of Hrk severely impairs the ability of *L. infantum* promastigotes to survive in the presence of edelfosine. Conservation of the antiapoptotic and proapoptotic activities of Bcl-X_L and Hrk, respectively, in *Leishmania* promastigotes may be considered an indication of the presence of BH3-bearing proteins, which have been implicated in the regulation of cell death, in the *Leishmania* proteome.

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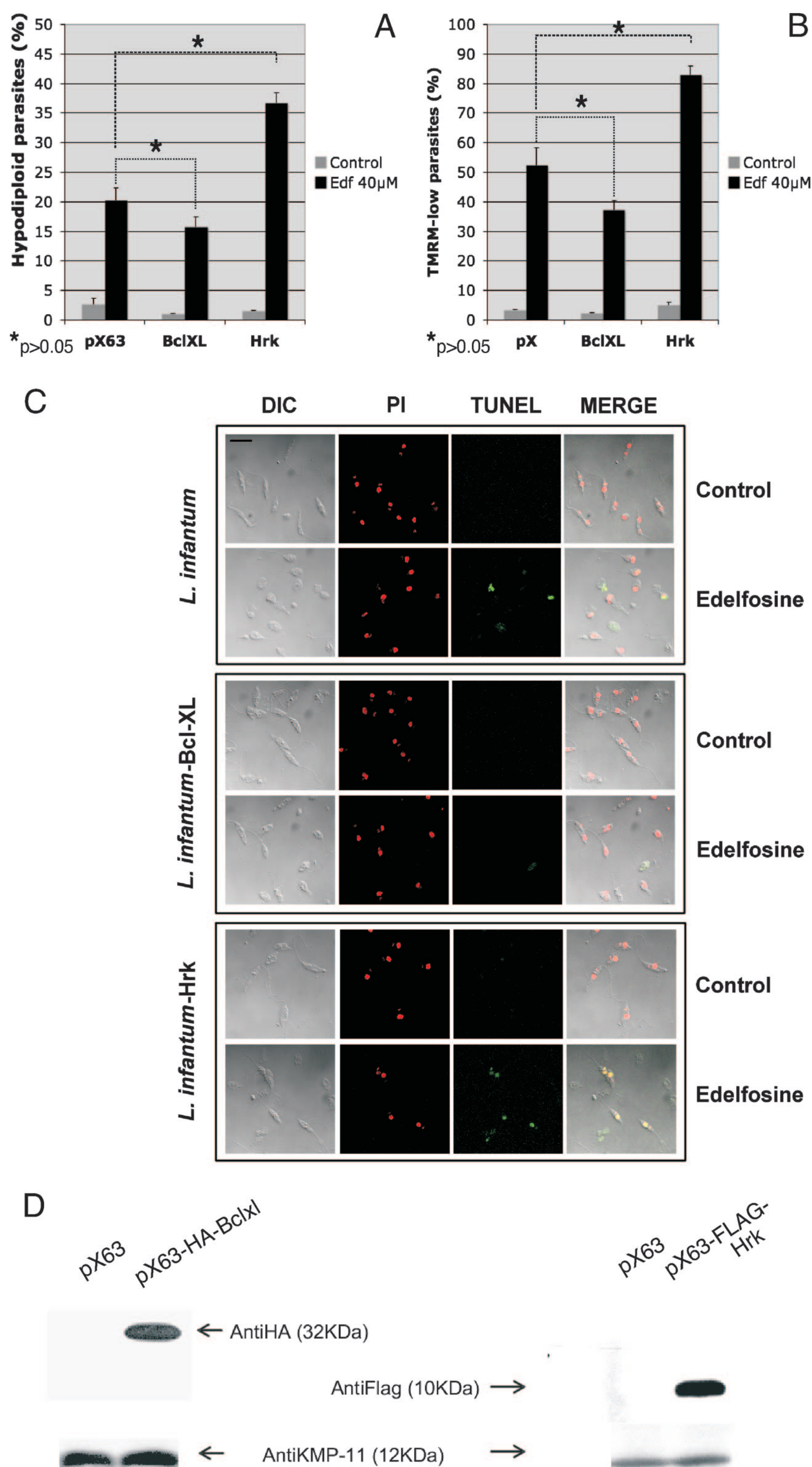

FIG. 2. Proteins from the Bcl-2 family modulate edelfosine-induced cell death in *L. infantum* promastigotes. Transfected *L. infantum* strains carrying the empty vector (pX63) or the genes encoding the antiapoptotic Bcl-X_L (BclXL) or proapoptotic Hrk were exposed to 40 μM edelfosine for 24 h. Cell death was measured in terms of DNA degradation (A) and the decrease in the mitochondrial membrane potential (B). Light gray bars, untreated parasites; black bars, edelfosine (Edf)-treated parasites; lines above the bars, standard deviations ($n = 3$); *, $P < 0.05$. (C) Confocal microscopy images of *L. infantum* strains transfected with empty pX63 (*L. infantum*), pX63–*bcl-X*$_L$ (*L. infantum*-Bcl-XL), or pX63-*hrk* (*L. infantum*-Hrk) and incubated in the absence of edelfosine (control) or exposed to 40 μM edelfosine for 24 h and then analyzed by the TUNEL assay, PI staining, and differential interference contrast (DIC). (D) Western blot analysis of the *Leishmania* strains expressing either Bcl-X_L or HrK. Detection of the ectopic proteins expressed was carried out with anti-HA (Bcl-X_L) or anti-FLAG (Hrk) antibodies. Antibodies against the Kmp-11 protein were used to confirm that the same amount of protein was loaded in each lane.

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This work was supported by grants FIS-PI021052, FIS-FEDER 04/0843, SAF 2006-12713-CO2, and CAM-UAH2005/035; by the Fundación de Investigación Médica Mutua Madrileña; by the Fundación la Caixa (grant BM05-30-0); and by a research grant from the Vicerectoría de Investigación, Universidad de Antioquia.

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